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PROTOCOL FOR INITIAL PURIFICATION OF BACTERIOCIN

by
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February 2013 – August 2014

U.S. Army Natick Soldier Research, Development and Engineering Center
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14. ABSTRACT

This report describes a protocol designed to instruct on how to partially purify an unknown bacteriocin to allow for rapid evaluation of its potential as an effective antimicrobial, and it briefly summarizes the effort by the Natick Soldier Research, Development and Engineering Center (NSRDEC), between February 2013 and August 2014, to develop the protocol. Bacteriocins are narrow-spectrum toxins that control pathogenic bacteria by out-competing them for resources while leaving beneficial bacteria unaffected. One of the limiting factors for the commercialization of bacteriocins is the time consuming and costly process for evaluating an unknown a bacteriocin for its potential effectiveness, which involves obtaining an ultra pure material and then characterizing and evaluating the bacteriocin. The aim of the NSRDEC effort was to develop a purification protocol that could be applied to each isolated bacteriocin to purify the material in sufficient quantities and to a sufficient purity to make initial evaluations. The new NSRDEC protocol includes filtering the lysate/filtrate through filters with a 30,000 molecular weight cut-off (MWCO), 10,000 MWCO, and 1,000 MWCO pore size to remove unwanted protein/debris. These filtering steps are performed before the extract is loaded onto the column. Evaluation of the new protocol indicated the filters significantly increased the purity to a level sufficient to allow essential assessment of the bacteriocin's potential for use as an effective antimicrobial.

15. SUBJECT TERMS

FILTERS	OPTIMIZATION	CELLS(BIOLOGY)	TEST AND EVALUATION
PEPTIDES	PREPARATION	SOLUBLE EXTRACTS	COLUMN CHROMATOGRAPHY
BACTERIA	PURIFICATION	CHARACTERIZATION	CELLULAR EXTRACTS(BIOLOGY)
PROTOCOLS	BACTERIOCINS	CULTURES(BIOLOGY)	
EXTRACTION	DESALINATION	COMMERCIALIZATION	

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PROTOCOL FOR INITIAL PURIFICATION OF BACTERIOCIN

1. Introduction

This report describes a protocol designed to instruct on how to partially purify an unknown bacteriocin to allow for rapid evaluation of its potential as an antimicrobial. This protocol was developed by the Natick Soldier Research, Development and Engineering Center (NSRDEC) between February 2013 and August 2014.

The current approach for combating pathogenic bacteria is to utilize broad-spectrum antimicrobials to kill the pathogenic bacteria by targeting most bacteria, including beneficial bacteria. Resistant bacteria strains survive. Over a period of repeated use, the resistant strains become the prevalent species, making the antimicrobial ineffective.

Recent studies have shown that many beneficial bacteria contribute to keeping pathogenic bacteria controlled by out-competing them for resources. Bacteriocins are narrow-spectrum toxins that act upon competing species of bacteria, leaving beneficial bacteria unaffected. Though bacteriocins have been investigated for decades, few have made it out of the lab. This is due in part to the time necessary to evaluate an unknown bacteriocin for potential use. Much of this time involves obtaining a sufficiently pure material for critical assessment. Traditionally, purification schemes are designed individually for each bacteriocin. The material obtained through these schemes is ultra-pure, but the approach is time-consuming and costly. It would be advantageous to have a purification protocol that could be applied to each isolated bacteriocin to purify the material in sufficient quantities and to a sufficient purity to make initial evaluations. This is the primary aim of this protocol.

2. Development of Protocol

The initial work by NSRDEC involved evaluating procedures for purification of different bacteriocins published in the literature. Comparison of the different schemes indicated several common steps. A draft purification protocol was derived from these steps, which included four steps: optimization, cell lysate/extract preparation, column purification, and a desalting. The peptide was tracked throughout the process using a soft agar overlay activity test.

Multiple bacteriocin-producing bacteria species were run through the initial protocol to evaluate its ability to produce a purified peptide. The results showed that, though the peptide was cleaner than a crude lysate/extract, it was not at the purity level needed for assessing potential use in future studies. Going back to the literature evaluation, it was determined that adding a filter step before loading the peptide onto a column would remove much of the contaminating extracellular debris to yield a cleaner material.

A new protocol was designed, which included filtering the lysate/filtrate through filters with a 30,000 molecular weight cut-off (MWCO), 10,000 MWCO, and 1,000 MWCO pore size to remove unwanted protein/debris. These filtering steps were added before the extract was loaded onto the column. Evaluation of the new protocol indicated the filters significantly increased the purity to a level sufficient to allow essential assessment of a bacteriocin's potential as an antimicrobial. Purified material was evaluated for yields, purity, minimal inhibitory concentration (MIC), and stability.

3. Summary and Scope of Test Method

This protocol involves initially evaluating the producer bacteria for optimal bacteriocin production on different media and under different conditions. Once the optimal conditions are

determined, cell lysates/extracts are prepared and filtered through a 0.22-µm filter to remove any cell debris. The filtrate is further filtered through a 30,000, 10,000 and 1,000 MWCO filter using tangential flow filtration. Retentates and filtrates are tested for activity, and the active fraction(s) is (are) loaded onto an equilibrated Diethylaminoethyl (DEAE) Sephadex A-25 column. The peptide is eluted using 350-mM and 1-M Sodium Chloride (NaCl). The flow through, wash, and elutions fractions are tested for activity. The active fraction is evaluated for purity, peptide concentration, peptide size, peptide stability, and MIC.

Activity at each stage is determined using the soft agar overlay method. Fields of the target organism are created by inoculating soft agar with the 4-h culture of the bacteria and pouring it over a media plate. Cell extracts or bacteriocin preps are dropped onto the field and incubated overnight. Positives are determined by the presence of a zone of inhibition around the drop. Samples are concentrated using a speed-vac to 1/10 concentration if the activity is low or absent.

Purity of the peptides is determined using a Bis-Tris PAGE gel. The peptide band is identified using a soft agar overlay of the gel. Peptide concentration is determined using the Bichronic Acid (BCA) protein assay. MIC assay is determined using the microtiter variation, with inhibition assessed on a spectrophotometer, measuring optical density (OD) at 600 nm. Stability is determined by incubating aliquots of the peptide at different temperatures and testing for retained activity at different times using the soft agar overlay method.

4. Apparatus, Reagents, and Materials

- *Incubator*
- *Sterilizer*
- *Shaker incubator*
- *Agar plates* – Use a combination of Nutrient, Tryptic Soy, Brain Heart Infusion (BHI), Mueller Hinton II, and Luria Broth (LB) plates. Prepare each as directed.
- *Broth* – As with the plates, prepare broth as directed for use with liquid cultures.
- *Centrifuge* – Run at 10,000 rpm for 30 min to remove cells and cellular debris from cell lysates/extracts.
- *Tangential flow* – It is the process used to filter the samples, and it includes washing the retentates with 25mM Tris buffer pH 8.5 and washing the filters with MQ-water and 0.2 mM of Sodium Hydroxide (NaOH), and storing the filters in 0.1 N Phosphoric acid.
- *Tris buffer* – Prepare 1 L of 25 mM Tris-HCl. Bring to pH 8.5 using NaOH and Hydrochloric acid (HCl). When needed for column elution, add NaCl was added to the buffer to increase the overall salt molarity.
- *DEAE Sephadex* – Equilibrate column using the 25mM Tris buffer. After elution, wash the column with 2M NaCl and 0.1M NaOH as needed.
- *Bis tris PAGE gel* – Prepare using either (1) 40% acrylamide, Temed, Sodiumdodecyl sulfate (SDS) and ammonium persulfate as a 10-12% gel or (2) purchased pre-poured as a 4-12% gel. Prepare samples with sample buffer and load as 20-µL quantities into the wells.
- *Simplyblue Coomassie stain* – Purchase from Invitrogen. Prepare gels as described in stain procedures provided by the vendor.
- *Eon microtiter plate reader* – Is used for MIC and BCA assay.
- *BCA reagent*
- *Biological safety cabinet* – Is required when using Class II or higher microorganisms.

5. Test Organisms

- Target bacteria – *Bacillus anthracis Sterne, Staphylococcus aureus* ATCC 27217*, or Pseudomonas aeruginosa CD81*. Store in a -80 ºC freezer.
- Bacterial isolate – Isolates from clinical and/or environmental sources. Store as a glycerol stock in a -80 ºC freezer.

6. Procedures

6.1. Prepare Target Bacteria:

- Inoculate a single colony of a fresh culture of *Bacillus anthracis Sterne, Staphylococcus aureus, or Pseudomonas aeruginosa* from a streak plate into 10 mL of culture broth.
- Incubate the culture on a shaker incubator set at 37 °C for 4 h, or until the OD measures 1 absorbance unit at 600 nm.
- Add 40 µl of this culture to 7 mL of soft agar, and pour the agar over the media.
- Once the soft agar hardens, place 6-uL drops of sample on to the soft agar, and allow the drops to absorb into the agar.
- Incubate the plate overnight at 37 °C.

6.2. Prepare Sample for Purification or Optimal Media Screening:

- Inoculate a single colony of a fresh culture of the test isolate from a streak plate into 10 mL of broth culture.
- Incubate the culture on a shaker incubator set at 37 °C for 4 h to an OD of 1.
- Inoculate a 1-L flask of broth with this culture if running a purification test, or inoculate plates with the culture for use with the optimal media test.

6.3. Determine Optimal Media:

- Prepare rectangular media plates*:*
  - Fill each plate with about 30 mL of sterile media.
  - Inoculate about 12 mL of soft agar with 20-40 µL of the prepared target organism culture (Section 6.1), and pour it over the media plate. If using mitomycin C, add 1.6 µL of the mitomycin stock to the soft agar before pouring onto the media. (The soft agar and media should be the same.)
  - Allow the soft agar to harden.
- Test the samples*:*
  - For each isolate, drop 6 µL of the 10-mL culture used in preparing the target organism (Section 6.1) onto the plate. Test duplicates at two different locations on each plate to confirm results for each media type, and test multiple isolates on a single plate.
  - Incubate each plate at either 30 or 37 ºC overnight. Evaluate activity by the presence of a zone of inhibition and by growth of the isolate.

6.4. Purify the Bacteriocin:

- Incubate the 1-L culture sample prepared for purification (Section 6.2) at the optimum temperature, with agitation, for 16 h.
- Remove the cells via centrifugation, and filter the supernatant through a 0.22-µm filter.

- Filter and test the filtrate:
  - Filter the filtrate through a 30,000 MWCO filter using the tangential flow, and wash the retentate twice with 200 mL of tris buffer (which, at this point, should reduce the filtrate to about 150 ml).
  - Filter the filtrate through a 10,000 MWCO filter, and again wash the retentate twice with tris buffer.
  - Filter the filtrate through a 1,000 MWCO filter, and again wash the retentate twice with tris buffer.
  - Test all filtrates and retentates for activity, both in an un-concentrated and concentrated form.
- Perform an MIC on the semi-purified peptide using the microtiter plate procedure, to determine activity.
- Test the fractions:
  - Load the most filtered fractions demonstrating activity onto the DEAE Sephadex column, and collect the flow through.
  - Wash the column with 200 mL of tris buffer, and collect the buffer.
  - Elute the peptide using a step gradient with tris buffer containing 150, 250, and 350 mM or containing 1M NaCl.
  - Test each fraction for activity, both in an un-concentrated and concentrated form.
- Analyze the active sample for protein concentration using the BCA assay.

6.5. Determine the Purity:

- Load 20-µL samples of the active fractions onto Bis-tris PAGE. It is necessary to desalt those samples for 150-mM and 1-M fractions, by using dialysis or G10 sephadex columns, in order to prevent interference when the gel is run.
- Stain the gels with Simplyblue Coomassie stain, and destain them in water.
- Identify the correct band by washing the gel in water to remove SDS and placing it onto a petri dish.
- Inoculate a soft agar with the target organism, pour it over the gel, and incubate it overnight.
- Compare pictures of the stained and overlayed gel to identify the active peptide band as determined by a zone of inhibition.

6.6. Perform Stability Tests:

- Incubate aliquots of either crude or purified material at different conditions for a given period of time.
- Perform activity tests to determine stability.

This document reports research undertaken at the U.S. Army Natick Soldier Research, Development and Engineering Center, Natick, MA, and has been assigned No. NATICK/TR-16/002 in a series of reports approved for publication.